IDENTIFICATION OF CYP3A4 AS THE ENZYME INVOLVED IN THE MONO-N-DEALKYLATION OF DISOPYRAMIDE ENANTIOMERS IN HUMANS

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(Received January 11, 2000; accepted April 19, 2000)

This paper is available online at http://www.dmd.org

ABSTRACT:
To identify which cytochrome P-450 (CYP) isoform(s) are involved in the major pathway of disopyramide (DP) enantiomers metabolism in humans, the in vitro formation of mono-N-desalkyldisopyramide from each DP enantiomer was studied with human liver microsomes and nine recombinant human CYPs. Substrate inhibition showed that SKF 525A and troleandomycin potently suppressed the metabolism of both DP enantiomers with IC50 values for R(−)- and S(+)DP of <7.3 and <18.9 μM, respectively. In contrast, only weak inhibitory effects (i.e., IC50 > 100 μM) were observed for five other representative CYP isoform substrates [i.e., phenacetin (CYP1A1/2), sparteine (CYP2D6), tolbutamide (CYP2C9), S-mephenytoin (CYP2C19), and p-nitrophenol (CYP2E1)]. Significant correlations (P < .01, r = 0.91) were found between the activities of 11 different human liver microsomes for mono-N-dealkylation of both DP enantiomers and that of 6β-hydroxylation of testosterone. Conversely, no significant correlations were observed between the catalytic activities for DP enantiomers and those for the O-deethylation of phenacetin, 2-hydroxylation of desipramine, hydroxylation of tolbutamide, and 4'-hydroxylation of S-mephenytoin. Further evidence for involvement of CYP3A450s was revealed by an anti-human CYP3A serum that inhibited the mono-N-dealkylation of both DP enantiomers and 6β-hydroxylation of testosterone almost completely (i.e., >90%), whereas it only weakly inhibited (i.e., <15%) CYP1A1/2- or 2C19-mediated reactions. Finally, the recombinant human CYP3A4 showed much greater catalytic activities than seven other isoforms examined (i.e., CYP1A2, 2A6, 2B6, 2C9, 2D6, 2E1, and 3A5) for both DP enantiomers. In conclusion, the metabolism of both DP enantiomers in humans would primarily be catalyzed by CYP3A4, implying that DP may have an interaction potential with other CYP3A substrates and/or inhibitors.
metabolism of DP enantiomers and other representative substrates of distinct human CYP isoforms were reported elsewhere (Chiba et al., 1993; Echizen et al., 1994; Koyama et al., 1994). The amounts of human liver microsomes used for the incubation of each substrate differed 4-fold (i.e., equivalent to 0.025–0.1 mg of protein) because of differences in the catalytic activities of microsomes against the respective substrates. For instance, the incubation of S-mephenytoin was performed with microsomes equivalent to 0.1 mg of protein, whereas the incubations of the remaining substrates (i.e., DP, phenacetin, desipramine, and testosterone) were done with microsomes equivalent to 0.025 mg of protein. Because the assay sensitivity for DP was improved substantially as compared with that reported in our previous studies (Echizen et al., 1993, 1994) (see details under HPLC Assays of Experimental Procedures), the incubation of DP enantiomers was performed with microsomes equivalent to 0.025 mg of protein. Substrate concentrations used for assaying the catalytic activities for the above substrates were: 10 µM for phenacetin, desipramine, and tolbutamide; 100 µM for S-mephenytoin; 30 µM for testosterone; and 32 µM for DP enantiomers. According to the enzyme kinetic parameters obtained from our previous study (Echizen et al., 1994), the microsomal enzyme activities at 32 µM for both DP enantiomers were attributable largely to a high rather than low-affinity enzyme component. In addition, it has been shown that the microsomal catalytic activities assessed by phenacetin O-deethylation, desipramine 2-hydroxylation, tolbutamide hydroxylation, S-mephenytoin 4′-hydroxylation, and testosterone 6β-hydroxylation at the substrate concentrations used herein were shown to be attributable to CYP1A2, CYP2D6, CYP2C9, CYP2C19, and CYP3A, respectively (Waxman et al., 1990; Relling et al., 1990; Dahl et al., 1992; Tassaneeyakul et al., 1993; Goldstein et al., 1994). Because the mono-N-dealkylation of DP is not involved in the chiral carbon atom, the chirality of the parent enantiomers remains unaltered by the metabolism. Thus, R-(-) and S(+)MND are considered to be derived exclusively from R(-)- and S(+)DP, respectively.

The enzyme reaction was initiated by adding 50 µl of NADPH-generating system consisting of 20 mM glucose 6-phosphate, 5 mM NADP, 40 mM MgCl2, and 10 U/ml of glucose 6-phosphate dehydrogenase into the incubation mixture that was preincubated at 37°C for 1 min. The incubation mixture contained appropriate amounts of microsomes, 0.1 M sodium phosphate buffer (pH 7.4), 0.1 mM EDTA disodium, and the respective substrates. After an incubation of 250 µl of the final reaction mixture at 37°C in a shaking water bath for 30 to 60 min depending on the substrates, the enzyme reaction was terminated by adding 25 µl of 2 N HClO4 (for DP) or 100 µl of acetonitrile (for other substrates) into the incubation mixture. Incubation times used for the respective substrates were: 45 min for DP enantiomers, 20 min for phenacetin and testosterone, 30 min for desipramine and tolbutamide, and 45 min for S-mephenytoin. All experiments were performed in duplicate or triplicate.

Incubation conditions used for the DP enantiomer metabolism with microsomes obtained from genetically engineered HepG2 cells expressing one of the human CYP3A3, 3A4, and 3A5 were essentially similar to those used for the DP metabolism with human liver microsomes. DP enantiomers and racemate (10 µM each) were incubated separately with the microsomes obtained from HepG2 cells (equivalent to 0.5 mg of protein/ml or 12.1 and 40.7 pmol of P450/ml for CYP3A3 and 3A4, respectively). In addition, DP enantiomers were incubated with the microsomes prepared from uninfected HepG2 cells to determine whether they possess a constitutional catalytic activity for the drug.

Inhibition Study. The effects of coinuciation of relatively selective inhibitors or substrates of six distinct human CYP isoforms and of a nonselective CYP inhibitor on the microsomal metabolism of each DP enantiomer were studied separately. Representative inhibitors or substrates used were: phenacetin for CYP1A1/2 (Tassaneeyakul et al., 1993), sparteine for CYP2D6 (Gonzalez, 1990), tolbutamide for CYP2C9 (Relling et al., 1990; Srivastava et al., 1991), S-mephenytoin for CYP2C19 (Wrighton et al., 1993; Goldstein et al., 1994), p-nitrophenol for CYP2E1 (Koop, 1992; Patten et al., 1992), and troleandomycin for CYP3A (Thummel and Wilkinson, 1998). A nonselective CYP inhibitor used was SKF 525A (Schenken et al., 1972). Each DP enantiomer (32 µM) was incubated with and without one of the inhibitor or substrates at concentrations of 0.1, 1, 10, and 100 µM under the incubation conditions described earlier. The MND formation rates determined in the presence of the respective concentrations of inhibitors or substrates were compared with the control values determined with the incubation of DP microsomes were prepared by differential centrifugation as described in details elsewhere (Echizen et al., 1993). The protein content of each microsomal preparation was determined by the method of Lowry et al. (1951). The microsomal samples were aliquoted, frozen, and stored at −80°C until used.

Incubation Conditions. Incubation conditions used for the microsomal
enantiomers alone and expressed as the percentage of the corresponding control values.

For assessing the inhibitory potency of each CYP isoform-selective substrate or nonselective CYP inhibitor, their concentrations associated with 50% inhibition of the metabolism of the respective DP enantiomers as compared with the corresponding control values (i.e., IC50) were determined based on the concentration inhibition curves. No substrates or inhibitor were preincubated with NADPH-generating system before initiating the DP metabolism. Experiments were performed with three to four different microsomal preparations that were obtained from distinct human liver samples.

**Assays.** MND formed in the incubation mixture was assayed according to the HPLC-UV detection method reported elsewhere (Echizen et al., 1993) with minor modifications. Briefly, to each reaction-terminated incubation mixture, 50 μl of the internal standard solutions (equivalent to 1 μg of timolol) was added, and the resultant mixture was centrifuged at 10,000g for 5 min. The supernatant was passed through a 0.45-mm (pore size) filter membrane (Gelman Science, Tokyo, Japan), and 50 μl of the filtrate was injected into the HPLC system that consisted of a model L-6000 pump (Hitachi Ltd., Tokyo, Japan), a model 655A-20 automatic sample injector (Hitachi), a reversed-phase HPLC system that consisted of a model L-6000 pump (Hitachi Ltd., Tokyo, Japan), a model 655A-20 automatic sample injector (Hitachi), a reversed-phase HPLC system in which a chromato-Integrator (Hitachi), and the concentrations of the respective metabolites were quantified with the peak-height ratios against the respective internal standards.

The mobile phase was delivered at 0.7 to 1.0 ml/min, depending on the analyte size; Eicom, Kyoto, Japan), and a model 8000 UV absorbance detector (Tosoh, Tokyo, Japan) set at 200 nm. Column temperature was maintained at 30°C by a water circulator.

Metabolites of other CYP isoform-selective substrates were determined according to the HPLC-UV absorption methods reported elsewhere (Chiba et al., 1993). The 2-hydroxysdesipramine assay was performed according to the method of Koyama et al. (1993) except that the metabolite was detected by a UV absorption method. Briefly, internal standards were used which were: phenobarbital, proparonazine, chlorpromazine, phenobarbital, and nitrazepam for O-deethylphenacem (i.e., parachetemol), 2-hydroxysdesipramine, hydroxytubamamide, 4′-hydroxymephenytoin, and 6′-hydroxytestosterone, respectively. A reversed-phase HPLC column, CAPCELL PAK C18 AG 120 (250 × 4.6 mm internal diameter, 5-μm particle size; Eicom, Kyoto, Japan), and a model 8000 UV absorbance detector (Tosoh, Tokyo, Japan) set at 200 nm. Column temperature was maintained at 30°C by a water circulator.

Chromatograms were recorded by a model D-2500 Chromato-Integrator (Hitachi), and the concentrations of the respective metabolites were quantified with the peak-height ratios against the respective internal standards.

**Data Analysis.** Data are expressed as mean ± S.D. throughout the text. Correlations between the catalytic activities of human liver microsomes for the selective substrates of six distinct CYP isoforms and those for DP enantiomers were analyzed by the least-squares linear regression method. P < .05 was considered statistically significant.

**Results.**

**Assays.** Under the chromatographic conditions used in this study, no chromatographic peaks that might have interfered with the determination of MND enantiomers and the internal standard (i.e., timolol) were observed in the presence or absence of seven inhibitors or substrates. HPLC assays for the metabolites of the selective substrates of six CYP isoforms and those for DP enantiomers were performed with no possible interfering peaks (chromatograms are not shown). For all metabolites and the internal standards, the mean extraction recoveries from the incubation mixture containing human liver or recombinant microsomes were >95% with coefficients of variation of <6%. Linearity of the microsomal metabolism for the respective CYP substrates and DP enantiomers with regard to the amounts of protein and incubation times have been confirmed in our laboratory, and a part of the data were reported elsewhere (Chiba et al., 1993; Echizen et al., 1994). Results obtained from duplicated incubations did not differ >10% for all the samples. When the incubation was carried out without the NADPH-generation system, no appreciable formation of MND was observed for both DP enantiomers (data not shown).

**Substrate Inhibition Study.** The effects of coadministration of seven distinct inhibitors or substrates of CYPs on the MND formation from each of the DP enantiomers with human liver microsomes are shown in Fig. 1. The nonselective CYP inhibitor, SKF525A, inhibited the metabolism of both DP enantiomers in a concentration-dependent and enantioselective manner with mean IC50 values of 0.4 and 5.4 μM for R(-)- and S(+) -DP, respectively. The mean maximum inhibitory effects elicited by SKF525A for the R(-)- and S(+) -DP were 89 and 84%, respectively. In addition, a selective inhibitor for CYP3A, troleandomycin, potently inhibited the metabolism of both DP enantiomers in a concentration-dependent and enantioselective manner; the mean IC50 values were 7.3 and 15.5 μM and the mean maximum inhibitory effects were 83 and 74%, for R(-)- and S(+) -DP, respectively. In contrast, the remaining selective substrates for five CYP isoforms elicited only a weak, if any, inhibitory effect on the DP metabolism. None of them produced inhibitory effects equal or greater than 50% as compared with the respective control values within the concentration range studied. Phenacetin (10 μM) and sparteine (1 and 10 μM) slightly activated the metabolism of S(+) -
DP, whereas p-nitrophenol (10 μM) activated the metabolism of R(2)- and S(1)-DP (Fig. 1).

**Correlation Study.** There were significant (P < .01) correlations between the microsomal catalytic activity for the 6β-hydroxylation of testosterone and that for the mono-N-dealkylation of both DP enantiomers (r = 0.91 for both DP enantiomers) (Fig. 2). The linear regression lines for R(2)- and S(1)-DP were Y = 0.10 · X + 0.0040 and Y = 0.83 · X + 0.014, respectively. The y-intercepts for both DP enantiomers differed insignificantly from 0 (95% confidence intervals for R(2)- and S(1)-DP were −0.028 to 0.036 and −0.004 to 0.032, respectively). In contrast, no significant correlations were observed between the microsomal activities for the four selective substrates of human CYP isoforms and those for both DP enantiomers (Fig. 2).

**Immunoinhibition Study.** The anti-human CYP3A serum elicited a potent inhibitory effect on the microsomal metabolism of both DP enantiomers in a dose-dependent manner (Fig. 3): the maximum inhibitory effects produced by 5 μl of antiserum were 100 and 95% for R(2)- and S(1)-DP, respectively, as compared with the corresponding control values. In contrast, the maximum volume of the anti-CYP3A serum (i.e., 5 μl) elicited only weak inhibitory effects on the microsomal metabolism of phenacetin (i.e., 9%) and S-mephénytoin (i.e., 15%) as compared with the respective control values.

**Recombinant CYP Study.** Western immunoblot analysis showed that the microsomes obtained from HepG2 cells genetically engineered for expressing one of the three distinct human CYP3A isoforms exhibited a single polypeptide band with approximate molec-
ular weights being compatible with the CYP3A isoforms (data not shown). Immunoblot analysis for the microsomes prepared from the control HepG2 cells showed no protein band that cross-reacted with anti-rat CYP3A2 antibody, and the microsomes showed no reduced CO binding spectrum (data not shown). With the use of the current vaccinia virus-based expression system, levels of CYP isoforms expressed in HepG2 cells ranged from 10 to 20 pmol/mg of total cell lysate protein. Although the microsomes prepared from the HepG2 cells expressing human CYP3A3 and 3A4 showed a substantial mono-N-dealkylation activity for both DP enantiomers and racemate, CYP3A5 showed much less activity than CYP3A3 and 3A4 (Fig. 4) despite that these three CYPs demonstrated largely comparable catalytic activities for the 6β-hydroxylation of testosterone (i.e., 3.0–7.5 pmol/min/pmol of P450). Interestingly, the recombinant CYP3A3 and 3A4 showed a preferentially greater catalytic activity for $S(1)$- over $R(2)$-DP. As to six other recombinant CYP isoforms examined, only CYP 2C9 showed a small, albeit measurable, catalytic activity for both DP enantiomers (Fig. 4).

**Discussion**

This is the first attempt for identifying which CYP isoform(s) are responsible for the major metabolic pathway of DP enantiomers (i.e., mono-N-dealkylation) in humans. Using human liver microsomes and anti-CYP3A serum we revealed that the mono-N-dealkylation of both DP enantiomers is mediated primarily by CYP3A (Figs. 1–3). With

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**Fig. 2.** Correlations between catalytic activities toward five representative CYP isoform substrates and those for $S(1)$- and $R(2)$-DP determined with 11 distinct human liver microsomal preparations.

(○) and (●) represent the catalytic activities for $R(2)$- and $S(1)$-DP, respectively. Detailed experimental conditions (e.g., substrate concentrations and incubation times) are given under Materials and Methods. Note that significant correlations ($r = 0.91$, $P < .01$ for both DP enantiomers) were observed between the catalytic activity toward CYP3A specific substrate (i.e., 6β-hydroxylation for testosterone) and mono-N-dealkylation of both DP enantiomers. NS = not significant.

**Fig. 3.** Immunoinhibition study on the mono-N-dealkylation of the respective DP enantiomers by anti-CYP3A serum performed with human liver microsomes.

Note that substantial and nearly superimposable inhibitions in a concentration-dependent manner were observed not only for the mono-N-dealkylation of $R(2)$- and $S(1)$-DP (○ and ●, respectively) but also for 6β-hydroxylation of testosterone (□). In contrast, only few changes were observed for phenacetin O-deethylation (△) and 4β-hydroxylation of S-mephenytoin (◇) mediated by CYP1A2 and CYP2C19, respectively. Data are means of duplicate experiments.
performing at rather low DP concentrations (i.e., 10 and 32 μM) in vitro. The in vitro enzyme reactions with human liver microsomes exhibited a biphasic enzyme kinetic profile, as shown that the mono-N-dealkylation of DP enantiomers and racemate was reported by Chiba et al. (1994) and Tassaneeyakul et al. (1993) using recombinant human CYPs. Although macrolide antibiotics are metabolized selectively by CYP3A, some of them (e.g., troleandomycin and erythromycin) were demonstrated to form a stable nitrosoalkane complex with the heme moiety of CYP3A (Periti et al., 1992), thereby potently inhibiting the metabolism of many CYP3A substrates in humans (Thummel and Wilkinson, 1998). In contrast, the representative substrates for the five other human CYP isoforms (i.e., CYP1A2, CYP2D6, CYP2C9, CYP2C19, and CYP2E1) elicited little or only a weak inhibitory effect on the metabolism of both DP enantiomers. Taken together, the results obtained from the substrate inhibition study (Fig. 1) suggest that the CYP3A isoform(s) are likely to be responsible for the DP metabolism in humans.

There were significant (r = 0.91, P < .01) correlations between the microsomal activities for the mono-N-dealkylation of both DP enantiomers and that for the 6β-hydroxylation of testosterone (Fig. 2). In addition, the y-intercepts of the regression lines for both DP enantiomers did not differ significantly from 0. Because the microsomal activity for the 6β-hydroxylation of testosterone is a result of CYP3A (Waxman et al., 1988), the microsomal metabolism of both DP enantiomers appears primarily to be mediated by this CYP subfamily. In support of this contention, there were no significant correlations between the microsomal activities for the metabolism of DP enantiomers and those for four other distinct metabolic pathways representing the microsomal activities of CYP1A2, CYP2D6, CYP2C9, and CYP2E1, respectively (Fig. 2). Nonetheless, we cannot totally eliminate the possibility that certain CYP(s), which were not assessed in this study, may have a significant contribution to the metabolism of DP enantiomers.

The specific anti-CYP3A serum (Kitada et al., 1992; Nakasa et al., 1993) added to the human liver microsomes inhibited the mono-N-dealkylation of both DP enantiomers as well as 6β-hydroxylation of testosterone (Fig. 3). The immuno-inhibition curves for both DP enantiomers and testosterone were almost superimposable among each other and the microsomal activities of both substrates were almost completely abolished at the maximum volume (i.e., 5 μl) of the anti-CYP3A serum. In contrast, only small inhibitory effects (i.e., <15%) were observed on phenacetin-O-deethylation and S-mephenytoin 4′-hydroxylation (Fig. 3) by the maximum amount of the anti-CYP3A serum, indicating that the anti-CYP3A serum used in this study was specific for CYP3A and that the metabolism of both DP enantiomers would be mediated almost exclusively by this CYP subfamily. However, we should interpret the results of the immuno-inhibition study with some reservation because it is quite difficult, if not impossible, to exclude the possibility that the anti-CYP3A serum used might inhibit other human CYP(s) other than those examined herein (i.e., CYP1A2 and CYP2C19).

The in vitro study assessing the metabolic activities of the nine distinct recombinant human CYPs toward DP enantiomers demonstrated that CYP3A3 and 3A4 possessed (by far) greater catalytic activities than any other CYP isoforms, and that the metabolism of DP inhibitor, SKF525A (Schenkman et al., 1972), produced a potent inhibitory effect on the microsomal metabolism of both DP enantiomers (Fig. 1). In addition, no appreciable MND formation was observed from both DP enantiomers unless the NADPH-generation system was added to the microsomal incubation mixture. These findings suggest that CYP enzyme(s) are involved in the oxidative mono-N-dealkylation of both DP enantiomers with human liver microsomes. The observation that troleandomycin showed a potent inhibitory effect on the metabolism of both DP enantiomers is compatible with our previous finding that macrolide antibiotics are potent inhibitors for the in vitro mono-N-dealkylation of DP racemate (Echizen et al., 1993) and enantiomers (Echizen et al., 1994) with human liver microsomes. The observation that troleandomycin showed a potent inhibitory effect on the metabolism of both DP enantiomers is compatible with our previous finding that macrolide antibiotics are potent inhibitors for the in vitro mono-N-dealkylation of DP racemate (Echizen et al., 1993) and enantiomers (Echizen et al., 1994) with human liver microsomes. Although macrolide antibiotics are metabolized selectively by CYP3A, some of them (e.g., troleandomycin and erythromycin) were demonstrated to form a stable nitrosoalkane complex with the heme moiety of CYP3A (Periti et al., 1992), thereby potently inhibiting the metabolism of many CYP3A substrates in humans (Thummel and Wilkinson, 1998). In contrast, the representative substrates for the five other human CYP isoforms (i.e., CYP1A2, CYP2D6, CYP2C9, CYP2C19, and CYP2E1) elicited little or only a weak inhibitory effect on the metabolism of both DP enantiomers. Taken together, the results obtained from the substrate inhibition study (Fig. 1) suggest that the CYP3A isoform(s) are likely to be responsible for the DP metabolism in humans.

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![Figure 4](image-url)
with these two CYP isoforms was enantioselective: S(+)-DP was metabolized preferentially over R(-)-DP at therapeutically relevant substrate concentration (i.e., 10 μM) (Fig. 4). This finding was consonant with that obtained from the previous in vitro study performed with human liver microsomes (Echizen et al., 1994) and in vivo human studies (Lima and Boudoulas, 1985; Echizen et al., 1991). In addition, the finding that both DP enantiomers are catalyzed largely by the same CYP isoforms would explain the reason why the metabolic competition was observed between the DP enantiomers during the in vitro study performed with human liver microsomes (Echizen et al., 1994). The reason that CYP3A5 possessed much lower catalytic activity than CYP3A3 and 3A4 toward both DP enantiomers remains unknown. However, different catalytic properties toward endogenous and exogenous substrates among CYP3A isoforms (i.e., CYP3A4, 3A5, and 3A7) were reported by Ohmori et al. (1998). The CYP3A subfamily is known to be expressed most abundantly (i.e., from 10–60% of total CYPs) in human liver and to play a pivotal role in the oxidative metabolism of many clinically important drugs (Thummel and Wilkinson, 1998). Among the four distinct CYP3A isoforms so far cloned (i.e., CYP3A3, 3A4, 3A5, and 3A7), CYP3A4 would be a major isoform in adult humans. Although CYP3A5 is polymorphically expressed in only approximately 10 to 20% of the adult liver (Aoyama et al., 1998; Wrighton et al., 1990), CYP3A7 is expressed exclusively in the fetal liver (Komori et al., 1990). CYP3A3 appears to constitute a very minor form in human liver (Bork et al., 1989). Collectively, we are tempted to speculate that CYP3A4 would be the major CYP isoform responsible for the hepatic metabolism of DP enantiomers in the majority of adult humans and adult patients given DP as a racemate. 

Assuming that CYP3A4 is involved mainly in the hepatic metabolism of DP enantiomers, it can be anticipated that DP might be susceptible to a metabolic inhibition by certain CYP3A-selective inhibitors and/or substrates (e.g., erythromycin and ritonavir). In this context, it is of interest that there are clinical reports that these CYP3A-oriented metabolic inhibitors/substrates may give rise to cardiac and/or neurological adverse reactions (Ragosta et al., 1989; Paar et al., 1997; product information of Norvir, 1997). However, DP is metabolized preferentially over any of CYP3A inhibitors particularly in patients with impaired renal function or in geriatric patients. In this context, it also remains to be studied if the in vitro reconstituent CYP3A system would be a useful tool in forecasting an in vivo metabolic interaction between DP and other CYP3A substrates or inhibitors.

Acknowledgments. We thank K. Manabe and A. Saito for their technical assistance, Dr. E. Koyama for her useful comments and suggestions in the preparation of the manuscript, and Dr. T. Ohmori for his generous supply of the specific antibody against human CYP3A.


